

AMENDMENTS TO THE SPECIFICATION

Please **replace** paragraph [0016] with the following paragraph:

[0016] Figure 2A-D. Schematic representation of Col17A1 model constructs. Detailed structure of (Figure 2A) the COL17A1-lacZ target (lacZ-T1) for the β -gal model-system and of (Figure 2B) the Col17A1 mini-gene target (T2). The relative position of primers lac9F, KI-3R and KI-5R are indicated. (Figure 2C) Schematic diagram of a PTM for the β -gal test-system. (1; 2; 3) Detailed structures and sequences of the PTM1, 3 and 5 binding domains, respectively. (SEQ ID NOS: 24 - 28. (Figure 2D) Schematic diagram of PTMs used in the Col17A1 mini-gene system (1; 2; 3). Detailed structures and sequences of the PTM2, 4 and 6 binding domains, respectively. (SEQ ID NOS: 24 - 28. Abbreviations: BP; branch point, PPT: polypyrimidine tract, ss: 5' and 3' splices sites, BD: binding domain

Please **replace** paragraph [0017] with the following paragraph:

[0017] Figure 3A-C. The β -gal test-system shows accurate *trans*-splicing at the RNA level and restoration of β -gal protein function in 293T cells using Col17A1 intron 51 as a target. Figure 3A. Demonstration of *cis*- and *trans*-splicing in 293T cells using the β -gal test-system. One representative experiment of 5 experiments is shown. 30 ng and 300 ng of total RNA were used for the detection of *cis*- (left panel) or *trans*-splicing (right panel), respectively. Lane 1: Transfection experiment with vector alone. Lane 2: Transfection of LacZ-T1 alone. Lanes 3, 4, 5: Transfection of PTM1, 3 and 5 alone. Lane 6, 7, 8: Co-transfection of 2 μ g LacZ-T1 and 2 μ g of either PTM1, 3 or 5. Lane M: 100 bp DNA size marker. Figure 3B. Upper panel: DNA sequence of *cis*-spliced lacZ-T1 target mRNA showing the correct splicing between the 5' and 3' exon and two in frame stop codons (underlined). The splice junction is indicated by an arrow. (SEQ ID NO: 29) Lower panel: DNA sequence of *trans*-spliced mRNA showing the accurate

trans-splicing and replacement of the stop codons. (SEQ ID NO: 30) Figure 3C. Restoration of β -gal activity is increased with respect to the length of the binding domain. β -gal activity representing the average of four independent transfection experiments. Lysates from 293T cells transfected with 2 μ g of LacZ-T1, PTM3 and PTM5 alone, respectively or co-transfected with 2 μ g target (LacZ-T1) and 2 μ g of PTM; LacZ-T1 + PTM1: 95.73 U/mg (+ SD 30 U/mg) protein; LacZ-T1 + PTM3: 117.52 U/mg (+ SD 30 U/mg) protein. LacZ-T1 + PTM5: 328.94 U/mg (+ SD 50 U/mg) protein. (SD = standard deviation).

Please **replace** paragraph [0022] with the following paragraph:

[0022] Figure 8. Endogenous *trans*-splicing of Col17A1 pre-mRNA with PTM5. Sequence of correct endogenously *trans*-spliced product showing the splice junction between exon 51 with lacZ 3' exon (A) (SEQ ID NO: 31) and confirmation by restriction digestion of 226bp RT-PCR product with MseI resulting in two fragments of 168bp and 58bp (B).

Please **replace** paragraph [0027] with the following paragraph:

[0027] Figure 13. PLEC-PTM-6 for repair of the 1287ins3 mutation in plectin deficient patient cells.

Figure 14A-E. *Trans*-splicing strategy for COL17A1 Gene.

Figure 14A. PTM6 consists of Col17A1 binding domain 51, spacer element, branch point (BP) and poly pyrimidine tract (PPT) followed by exon 52-56 cloned into pcDNA3.1(-). This construct was transiently transfected into GABEB cells harboring the 4003 del TC mutation.

Figure 14B. Semi-nested RT-PCR with BPAG2 primer 51-1F, 53-1R and 52-1R. RNA was extracted from PTM6 transfected GABEB cells and semi-nested RT-PCR was performed with BPAG2-primer 51-1F, 53-1R and 52-1R. By sequencing the expected 323bp fragment heterozygosity of mutant and corrected alleles could be demonstrated. (SEQ ID NOS: 32-34).

Figure 14C. TOPO Cloning +Nla III digestion. For quantification of wildtype vs. mutant DNA in the 323bp fragment, it was cloned into a TOPO vector. 100 clones were analysed by colony PCR and subsequent NlaIII digest, which detects the 4003 del TC mutation in the COL17A1 gene. A given digest profile of 4 different possibilities and fragment sizes is shown.

Figure 14D. Sequencing of mutant and wildtype clones confirmed the correct *trans*-splicing of PTM6 into GABEB cells. Sequencing of mutant and wildtype clones confirmed the correct *trans*-splicing of PTM6 into GABEB cells. (SEQ ID NOS:35-38).

Figure 14E. Analysis of 100 clones revealed 48 clones with correct *trans*-splicing and correction of the mutation 4003 del TC in the COL17A1 gene.

Figure 15. Immunofluorescence of transfected PTM6 into GABEB cells with Lipofectamine Plus.

Figure 16 depicts trans-splicing strategy for COL7A1 gene (Dystrophic epidermolysis bullosa). Figure 16A. 3' *trans*-splicing in a LacZ-model system to target intron 72 of the COL7A1 gene. Figure 16 B. Schematic drawing of LacZ-Target1 (T1) containing COL7A1-Intron 72 cloned between LacZ 5' and stop-LacZ 3' in a pcDNA3.1 expression vector. LacZ-PTM consists of BD intron 72 cloned 5' of functional LacZ 3' in a pcDNA 3.1 expression vector.

Figure 17. β -gal Assay of a cotransfection of COL7 target T1 and PTM 8, 9, 10 in 293T cells with Lipofectamin.

Figure 18. β -gal staining of COL7PTM transfected human HEK 293T cells. left panel:(a) target +PTM8, (c) target +PTM9, (e) target =PTM10; right panel:corresponding control PTM without target.

Please **replace** paragraph [0037] with the following paragraph:

[0037] The PTM molecule may also contains a 3' splice region that includes a branch point sequence and a 3' splice acceptor AG site and/or a 5' splice donor site. The 3' splice region may further comprise a polypyrimidine tract. Consensus sequences for the 5' splice donor site and the 3' splice region used in RNA splicing are well known in the art (See, Moore, *et al.*, 1993, The RNA World, Cold Spring Harbor Laboratory Press, p. 303-358). In addition, modified consensus sequences that maintain the ability to function as 5' donor splice sites and 3' splice regions may be used in the practice of the invention. Briefly, the 5' splice site consensus sequence is AG/GURAGU (where A=adenosine, U=uracil, G=guanine, C=cytosine, R=purine and /=the splice site) (SEQ ID NO:1). The 3' splice site consists of three separate sequence elements: the branch point or branch site, a polypyrimidine tract and the 3' consensus sequence (YAG). The branch point consensus sequence in mammals is YNYURAC (Y=pyrimidine; N=any nucleotide) (SEQ ID NO:2). The underlined A is the site of branch formation. A polypyrimidine tract is located between the branch point and the splice site acceptor and is important for efficient branch point utilization and 3' splice site recognition. Other pre-messenger RNA introns beginning with the dinucleotide AU and ending with the dinucleotide AC have been identified and referred to as U12 introns. U12 intron sequences as well as any sequences that function as splice acceptor/donor sequences may also be used to generate the PTMs of the invention.

Please **replace** paragraphs [0046]-[0047] with the following paragraphs:

[0046] 5' fragment sequence (SEQ ID NO:3):

gtagttcttttgttcttcactattaagaacttaatttggtgtccatgtctcttttttctagtttgtagtgctggaaggtattttggagaaat
tcttacatgagcattaggagaatgtatgggtgtagtgcttgtataatagaaattgtccactgataatttactctagttttatttcctcatattttt
cagtggctttttctccacatctttatatttgcaccacattcaacactgtagcggccgc.

[0047] 3' fragment sequence (SEQ ID NO:4):

caactatctgaatcatgtgccccctctctgtgaacctctatcataatacttgcacactgtattgtaattgtctcttttactttcccttgatc
ttttgtcatagcagagtacctgaaacaggaagtattttaaatattttgaatcaaataagtgtaataagaatctttacaataagaatatacacttctgc
ttaggatgataattggaggcaagtgaatcctgagcgtgattgataatgacctaataatgatgggttttattccag

Please **replace** paragraph [0048] with the following paragraph:

[0048] In yet another specific embodiment of the invention, consensus ISAR sequences are included in the PTMs of the invention (Jones *et al.*, 2001 *Nucleic Acid Research* 29:3557-3565). Proteins bind to the ISAR splicing activator and repressor consensus sequence which includes a uridine-rich region that is required for 5' splice site recognition by U1 SnRNP. The 18 nucleotide ISAR consensus sequence comprises the following sequence:
GGGCUGAUUUUCCAUGU (SEQ ID NO:5). When inserted into the PTMs of the invention, the ISAR consensus sequences are inserted into the structure of the PTM in close proximity to the 5' donor site of intron sequences. In an embodiment of the invention the ISAR sequences are inserted within 100 nucleotides from the 5' donor site. In a preferred embodiment of the invention the ISAR sequences are inserted within 50 nucleotides from the 5' donor site. In a more preferred embodiment of the invention the ISAR sequences are inserted within 20 nucleotides of the 5' donor site.

Please **replace** paragraph [0067] with the following paragraph:

[0067] Alternatively, synthetic PTMs can be generated by *in vitro* transcription of DNA sequences encoding the PTM of interest. Such DNA sequences can be incorporated into a wide variety of vectors downstream from suitable RNA polymerase promoters such as the T7, SP6, or T3 polymerase promoters. Consensus RNA polymerase promoter sequences include the following:

T7: TAATACGACTCACTATAGGGAGA (SEQ ID NO:6)

SP6: ATTTAGGTGACACTATAGAAGNG (SEQ ID NO:7)

T3: AATTAACCCTCACTAAAGGGAGA (SEQ ID NO:8).

The base in bold is the first base incorporated into RNA during transcription. The underline indicates the minimum sequence required for efficient transcription.

Please **replace** paragraphs [0093]-[0094] with the following paragraphs:

[0093] Target construction. LacZ-T1 (Figure 2A) included a lacZ 5' "exon" (1-1788 bp) followed by intron 51 of the Collagen 17A1 gene (282 bp) and a LacZ 3' "exon" (1789-3174 bp). This lacZ 3' exon contained two stop codons at position 1800 bp. Intron 51 of Coll17A1 was amplified by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) using genomic DNA as template and primers:

Int51U (5'-CGGGATCCGTAGGTGCCCCGACGGTGATG-3') (SEQ ID NO:9);

and

Int51D (5'-CTAGGGTAACCAGGGTGAGAAGCTGCATGAGT-3') (SEQ ID NO:10).

[0094] The amplified product was digested with BamHI and BstEII (New England Biolabs, Beverly, MA) and inserted between the two lacZ exons. T2 (Figure 2B) included the genomic sequence of exon 51, intron 51 and exon 52 followed by a FLAG sequence. The genomic sequence of exon 51, intron 51 and exon 52 was amplified using Pfu DNA polymerase and primers:

COLI-F (5'-CTAGGCTAGCCTGCCGGCTTGTCATTCATCC-3') (SEQ ID NO:11) and

COLI-R (5'-CTAGAAGCTTTTACTTGTCATCGTCGTCCTTGTA
GTCGCTGCATGCTCTCTGACACC-3') (SEQ ID NO:12).

Please **replace** paragraph [0096] with the following paragraph:

[0096] Pre-trans-splicing molecules (PTMs). pCOL17-PTM1 (Figure 2C) was constructed by digesting PTM14 (Intronn Inc., Rockville, MD) with EcoRI and KpnI replacing the CFTR binding domain (Mansfield SG *et al.*, 2000 7:1885-95) with a 80 bp oligonucleotide containing a 32 bp antisense binding domain (BD), a 18 bp spacer, branch point (BP), a polypyrimidine tract (PPT), and an acceptor AG dinucleotide followed by a lacZ 3' exon (1789-3174 bp). The use of BP and PPT follows consensus sequences which are needed for performance of the two phosphoryl transfer reactions involved in *cis*-splicing and also in *trans*-splicing, pCOL17-PTM4 and pCOL17-PTM6 were constructed by digesting PTM1, 3, and 5 with KpnI and HindIII and replacing the lacZ 3' exon with the exon 52 to 56 cDNA sequence of COL17A1 (Figure 2D). The cDNA sequence was amplified with Pfu DNA polymerase from poly-dT primed cDNA using the following primers:

COL2-F (5'-CTAGGGTACCTCTTCTTTTTTTTGATATCCTGCA

GGTCCTGATGTGCGCAGC-3') (SEQ ID NO:13); and

COL-2-R (5'-CTAGAAGCTTTTATGGAGACCTTGGACCTAAG-3') (SEQ ID NO:14).

Please **replace** paragraph [0102] with the following paragraph:

[0102] Reverse Transcription Polymerase Chain Reaction. RT-PCR was performed using a SuperScript OneStep™ RT-PCR Kit (Life Technologies) according to the manufacturer's protocol. Each reaction contained 50 to 500 ng of total RNA and 100 ng of a 5'- and 3'-specific primer in a 25 µl reaction volume. RT-PCR products were separated by gel-electrophoresis using 2% agarose gels. Primers used to estimate the products of *cis* and *trans*-splicing were as follows:

LAC9F (5'-ATCAAATCTGTCGATCCTTCC-3') (SEQ ID NO:15); and

KI-3R (5'-GACTGATCCACCCAGTCCCATTA-3') (SEQ ID NO:16) for *cis*-, and LAC9F and

KI-5R (5'-GACTGATCCACCCAGTCCCAGAC-3') (SEQ ID NO:17) for *trans*-splicing. For position of these primers on the plasmids see Figure 2A. RT-PCR analysis for the COLI7A1-mini-gene *cis*-splicing was performed using the following primers:

Ex51-1F (5'-CATCCCAGGCCCTCCAGGAC-3') (SEQ ID NO:18); and FLAG-R (5'-TTGTCATCGTCGTCCTTGTAG-3') (SEQ ID NO:19), while Primers Ex51-1F and

KI-53-1R (5'-GTAGGCCATCCCTTGAC-3') (SEQ ID NO:20) were used for the detection of *trans*-splicing. For position of these primers on the plasmids see Figure 5B.

Please **replace** paragraph [0127] with the following paragraph:

[0127] Western Blot analysis. Confluent cells are washed with PBS and scraped off the plate. Cell pellets are lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton-X 100, 0.1% (w/v) SDS, 0.5 mM EDTA, 10 μ M leupeptin, 100 μ M phenylmethylsulfonylfluoride, 100 μ M DTT. The epidermis from organotypic culture is lysed directly. 20 μ g protein of control and test KC are loaded on a 5% SDS Polyacrylamide Gel. Following electrophoresis, proteins are transferred to nitrocellulose (Hybond C pure; Amersham Pharmacia Biotech, Little Chalfont, UK) in 48 mM Tris-HCl, 39 mM Glycine, 20% (v/v) MeOH, 0.037% (w/v) SDS. The primary monoclonal antibody 5B3-is diluted 1:3 in blocking buffer (200 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.2% (w/v) I-Block, 0.1% (v/v) Tween 20). Immunodetection is monitored with the Western-Star™ Chemiluminescent Detection System (Tropix Inc., Bedford, MA, USA) following the manufacturer's instructions.

Primers: PLEC-FN: 5' GGG AGC TGG TGC TGC TGC TGC TTC 3' (SEQ ID

NO:21)

PLEC-FM: 5' GGG AGC TGG TGC TGC TGC TGC TGC 3' (SEQ ID

NO:22)

PLEC-R: 5' CTC TCA AAC TCG CTG CGG AGC TGC 3' (SEQ ID

NO:23).

AMENDMENTS TO THE DRAWINGS

Please replace the drawings with the twenty seven (27) sheets of replacement drawings submitted herewith.

The attached twenty seven (27) sheets of drawings includes changes to Figure 1 to eliminate the text. Also included is a annotated sheet showing changes to Figure 1.

Attachment: Replacement drawings (Figures 1-18)

Annotated sheet of Figure 1